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Transcription factor Reb1 is required for proper transcriptional start site usage at the divergently transcribed *TFC6-ESC2* locus in *Saccharomyces cerevisiae*.

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Abbreviations: Pol, RNA polymerase; NDR, nucleosome depleted region; NFR, nucleosome free region; TSS, transcription start site.

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ABSTRACT

Eukaryotic promoters generally contain nucleosome depleted regions near their transcription start sites. In the model organism *Saccharomyces cerevisiae*, these regions are adjacent to binding sites for general regulatory transcription factors, and the Reb1 protein is commonly bound to promoter DNA near such regions. The yeast *TFC6* promoter is a unique RNA polymerase II promoter in that it is autoregulated by its own gene product Tfc6p, which is part of the RNA polymerase III transcription factor complex TFIIIC. We previously demonstrated that mutation of a potential Reb1 binding site adjacent to the TFIIIC binding site in the *TFC6* promoter modestly reduces transcript levels, but leads to a severe decrease in Tfc6 protein levels due to an upstream shift in the *TFC6* transcription start site. Here we confirm that Reb1p indeed binds to the *TFC6* promoter, and is important for proper transcription start site selection and protein expression. Interestingly, loss of Reb1p association at this site has a similar effect on the adjacent divergently transcribed *ESC2* promoter, resulting in a significant increase of 5'-extended *ESC2* transcripts and reduction of Esc2 protein levels. This altered divergent transcription may be the result of changes in nucleosome positioning at this locus in the absence of Reb1p binding. We speculate that an important function of general regulatory factors such as Reb1p is to establish and maintain proper transcription start sites at promoters, and that when binding of such factors is compromised, resulting effects on mRNA translation may be an underappreciated aspect of gene regulation studies.

1. Introduction

Active eukaryotic RNA polymerase II (Pol II) promoter regions within cells are characterized by nucleosome depleted regions (NDRs) in the vicinity of transcription start sites (TSS). In the budding yeast *S. cerevisiae*, this fact has been demonstrated by both *in vivo* and *in vitro* genome-wide nucleosome mapping studies (YUAN *et al.* 2005; MAVRICH *et al.* 2008a; KAPLAN *et al.* 2009; NAGARAJAVEL *et al.* 2013), and further analysis confirmed a similar architecture in metazoans (MAVRICH *et al.* 2008b). Nucleosomes adjacent to promoter NDRs, referred to as the -1 and +1 nucleosomes, appear to be relatively well positioned within populations of cells, and are thought to influence the transcriptional start site (JIANG AND PUGH 2009). Passive positioning of these promoter-proximal nucleosomes appears to be determined both by intrinsic underlying DNA sequences and nearby DNA bound proteins (KAPLAN *et al.* 2009; ZHANG *et al.* 2009), and maintenance of NDRs appears to involve active processes utilizing chromatin remodelers such as the RSC and ISW2 complexes (BADIS *et al.* 2008; HARTLEY AND MADHANI 2009; YEN *et al.* 2012; GANGULI *et al.* 2014).

In *S. cerevisiae*, several general regulatory factors (GRFs) have been directly implicated in contributing to NDR formation, and include Abf1, Cbf1, Rap1, Reb1, and Tbf1 proteins (KENT *et al.* 1994; YARRAGUDI *et al.* 2004; BADIS *et al.* 2008; HARTLEY AND MADHANI 2009; TSANKOV *et al.* 2010; GANAPATHI *et al.* 2011; TSANKOV *et al.* 2011). Reb1p is a sequence-specific DNA binding protein containing two myb-like regions, and is essential for viability in *S. cerevisiae* (MORROW *et al.* 1989; JU *et al.* 1990; MORROW *et al.* 1990; MORROW *et al.* 1993). Reb1p can bind to sites within transcriptional control regions of genes transcribed by either RNA polymerase I or RNA

polymerase II (CHASMAN *et al.* 1990; MORROW *et al.* 1990; WANG *et al.* 1990), recognizing a consensus YYACCCG sequence (LIAW AND BRANDL 1994). Numerous studies have revealed that Reb1p is involved in the regulation of transcription by acting as a weak activator (BRANDL AND STRUHL 1990; CHASMAN *et al.* 1990; REMACLE AND HOLMBERG 1992). Furthermore, Reb1p was demonstrated to bind the terminator of the rRNA transcription unit, and was thought to be involved in the termination of transcription by RNA polymerase I (LANG AND REEDER 1993), however further studies demonstrated that this function is mediated by another myb-domain protein Nsi1p (REITER *et al.* 2012). Recently, Reb1p was uncovered to have a roadblock function that terminates progressing Pol II transcription, which in turn restricts pervasive cryptic transcription and readthrough transcription in the yeast genome (COLIN *et al.* 2014). Also, Reb1p was reported to interact with the RSC (remodels the structure of chromatin) complex, and is involved in the formation of nucleosome free regions (NFR) (RAISNER *et al.* 2005; WIPPO *et al.* 2011).

Previous results from our lab demonstrated that the *TFC6* promoter is autoregulated by its own gene product Tfc6p, which is one component of the six-polypeptide RNA polymerase III (Pol III) transcription factor complex TFIIIC (KLEINSCHMIDT *et al.* 2011). Forced overexpression of Tfc6p increased *in vivo* binding of the entire TFIIIC complex to the *TFC6* promoter (at its binding site referred to as *ETC6*, extra TFIIIC-6), and overexpression also resulted in decreased expression of a marker gene driven by the *TFC6* promoter. This Tfc6p mediated Pol II regulation was unexpected, as TFIIIC predominately regulates Pol III promoters, and this was a unique finding of a Pol III transcription factor directly regulating a Pol II promoter. The same

study demonstrated that mutation of the *TFC6* promoter just upstream of *ETC6* (encompassing a sequence identical to the Reb1p consensus binding site) results in a slow growth phenotype that is rescued by episomal expression of Tfc6 protein, and results in ~50% reduction in *TFC6* mRNA levels (KLEINSCHMIDT *et al.* 2011). More detailed analysis of yeast containing this Reb1p binding site mutation demonstrated that a 5'-extended *TFC6* mRNA was produced, which led to an approximately 15-fold reduction in Tfc6 protein levels (WANG *et al.* 2014).

Based on our previous work indicating that ectopic overexpression of Tfc6p leads to increased TFIIIC complex binding to *ETC6* and downregulation of *TFC6* promoter activity, and given the fact that an apparent Reb1p consensus binding site is located just upstream of the *ETC6* site, we wanted to verify whether Reb1p actually binds to the *TFC6* promoter, and how both Reb1p and TFIIIC complex binding mediate regulation of *TFC6*. Both *in vivo* and *in vitro* experiments described here demonstrate that Reb1p indeed binds to and is required for normal *TFC6* expression, and while it does not appear to be involved in autoregulation, Reb1p activity affects normal transcription start site selection at *TFC6*. Metabolic depletion of Reb1p phenocopies the *TFC6* promoter mutant in that the depleted cells contain increased levels of *TFC6* mRNA with an extended 5'-end, and also show reduced Tfc6 protein levels under Reb1p depleted conditions. Interestingly, loss of Reb1p binding to this promoter site also results in a 5'-extension and reduced translation of the divergently transcribed *ESC2* gene. Finally we show that nucleosome occupancy within this intergenic region is altered when Reb1p binding is compromised, which may contribute to the observed alteration in transcription start site usage.

2. Materials and methods

2.1 Yeast strains and growth media

All yeast strains were generated from the W303-1a background. The construction of Reb1 consensus binding site mutant strain DDY4300, containing a 12-bp mutation in the *TFC6* promoter, was described previously (KLEINSCHMIDT *et al.* 2011). 3X-FLAG epitope tagged strains used for Western blot analysis were constructed by amplifying plasmid P3-FLAG-KanMX (GELBART *et al.* 2001) with ~65bps flanking sequence on each side of the stop codon of the target gene. Specific yeast strains for Reb1p depletion were constructed by integrating the *GAL1* promoter upstream of the *REB1* open reading frame. Plasmid pFA6a-KanMX6-p*GAL1* (LONGTINE *et al.* 1998) was amplified with primers DDO1793/1794 containing 65 bps of homology upstream of the *REB1* start codon and 63bps homology downstream of the start codon, attached with 20 bp homology to either end of the KanMX-*GAL1* promoter cassette. The PCR product was purified and transformed into a diploid yeast strain DDY5240 that was previously engineered to contain a 9X-Myc-*TRP1* epitope tag at the end of both copies of the Reb1 coding sequence using plasmid pYM6 (KNOP *et al.* 1999) and oligos DDO-1591/1592. Transformed colonies were selected on YPGal plates containing 200µg/ml geneticin. Resistant colonies were confirmed by PCR with primer sets DDO-466/1797 and DDO-1797/RR00013. Positive PCR products were further confirmed by sequencing, and the corresponding strains were sporulated to obtain haploids. G418 resistant haploids were re-confirmed by PCR with the same primer sets. Analogous nourseothricin resistant strains were constructed by transforming the KanMX strains with a PCR product

amplified from the NatMX6-pGAL cassette in plasmid pFA6a–NatMX6-p*GAL1* (HENTGES *et al.* 2005; VAN DRIESSCHE *et al.* 2005) using oligos SWAP F and SWAP R to interchange the marker genes.

Yeast cultures were grown in nutrient rich YPD media (1% yeast extract, 2% peptone, and 2% dextrose). *GAL* promoter conditional strains were grown in YPGal, and shifted to YPD for depletion. Colony growth assays in figure 1 were performed on YMD agar plates (yeast nitrogen base, U.S. Biologicals #Y2025) with the indicated dropout supplements. All yeast cultures were grown at 30°C.

2.2 In vivo reporter assays of Reb1p function at the TFC6 promoter

HIS3 marked plasmids expressing either 3X-FLAG-*REB1* or 3X-FLAG-*NSI1* driven by the *ADH1*-promoter or empty *ADH1*-promoter vector were transformed into yeast strain DDY4521 (KLEINSCHMIDT *et al.* 2011). This diploid strain has one copy of the *TFC6* open reading frame (ORF) replaced with the *URA3* ORF as a reporter gene, and cell growth on the minimal medium lacking uracil was used as an indicator of *TFC6* promoter activity. After transforming with each plasmid and selecting on media lacking histidine, cultures were grown in liquid media and plated at approximately 100 colony forming units per plate on medium lacking either histidine or lacking both histidine and uracil. Relative colony size was measured using ImageJ software (http://imagej.nih.gov/ij/).

2.3 Recombinant Reb1p DNA binding domain expression and purification

The *REB1* DNA binding domain coding sequence was cloned (essentially as described in Morrow et al., 1993) into pET30A(+) (Novagen) to include an N-terminal His-tag for overexpression and purification in *E. coli*. The targeted *REB1* sequence was amplified from full length *REB1* plasmid pDD1252 with primers DDO1510/1511 including *Nco*I and *Bam*HI restriction sites, and cloned into pET30A(+) cut with the same enzymes. BL21 *E. coli* cells containing the resulting plasmid were grown in 1L 2XYT media in a shaker at 37°C to mid-log phase (A600= ~1.0). Protein induction was performed by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside) to 1mM and incubated for another 6 hours before collecting cells by centrifugation and freezing at -20°C. Cell pellets were thawed and resuspended in 25ml lysis buffer (20mM HEPES pH 8, 500mM NaCl, 0.1mM EDTA, 0.1% Triton X-100, 0.1mM PMSF) and sonicated on ice 5 X 15 seconds using a Branson Model 250 sonicator equipped with a microtip and amplitude set to 70%. Triton X-100 was added to 1% and the cell suspension was incubated with gentle rocking for 30 minutes at 4°C. The cell lysate was clarified by centrifugation at 10,000xg for 10 minutes at 4°C. Cobalt beads (Clontech TALON resin, #635502) were washed with lysis buffer before adding to the cell lysate, and the mixture was gently rocked at 4°C for 30 minutes. Then, beads were gently pelleted (~4500xg) and washed twice with wash buffer (20mM HEPES pH 8, 500mM NaCl, 0.1mM EDTA, 0.1% Triton X-100, 0.1mM PMSF, 5mM imidazole). Reb1 protein was eluted three times at 4°C with 1ml elution buffer (250mM imidazole, 100mM Tris pH 8.0, 120mM NaCl) and all eluates were pooled. Purified Reb1 protein was dialyzed in 1L HEMG buffer

(25mM HEPES pH 7.6, 0.2M EDTA, 12.5mM $MgCl_2$, 10% glycerol, 0.1mM PMSF) overnight at 4°C. Aliquots were stored at -80°C.

2.4 Gel-shift assays

Purified Reb1 protein was incubated with three different sets of ^{32}P-end labeled synthetic oligonucleotides (DDO1512-1513: rDNA NTS2 region; DDO1514-1515: *TFC6* promoter; DDO610-611: yeast *YTA7* open reading frame). The labeled DNA probes were incubated with increasing amounts of purified Reb1p DNA binding domain (0ng, 200ng, 500ng) in a total volume of 20µl binding reactions (10mM Tris pH 7.5, 50mM NaCl, 5mM $MgCl_2$, 1mM EDTA, 1mM DTT, 5% glycerol, 100µg/ml BSA). 1µg of non-specific competitor DNA (poly dG:dC) was added into the reaction. For each probe, competition was performed by adding 100 fold excess unlabeled probe into a 500ng protein reaction. 15µl of each reaction was loaded onto 5% acrylamide gel and run at 80 volts for 30 minutes in 0.5X TBE. Gels were dried and exposed on a phosphorimager cassette for 3 hours to overnight.

2.5 Reb1p depletion and Western blotting

KanMX-p*GAL1-REB1* strain DDY5248 was grown in YPGal + G418 (200 µg/ml) medium at 30°C until the O.D. 600 reached ~1.0. 25ml aliquots were processed for protein extraction and RNA extraction before switching the remaining culture into YPD media to shut off the *GAL1* promoter and deplete Reb1p. Aliquots were collected at various time points after switching to dextrose (2h, 4h, 8h, and overnight) and washed 1X in 1ml ice-cold 1X PBS, and cell pellets were stored at -80°C in screw-capped microfuge

tubes. Pellets were thawed and resuspended in ~150µl of lysis buffer (20mM Tris-Cl 7.2, 125mM KAc, 4mM $MgCl_2$, 0.5mM EDTA, 5mM Sodium Bisulphite, 0.1% Tween-20, 12.5% Glycerol, 1mM DTT, 2µg/ml Leupeptin, 2µg/ml Pepstatin A, 1mM PMSF). 300 µl glass beads were added, and the suspension was agitated at 4°C for 15'' X 5 at maximum speed in a bead beater (Mini-Beadbeater, Biospec). Lysates were clarified by centrifugation at 15,000xg at 4°C in a refrigerated microfuge, and protein concentrations were determined using the Bradford assay (Bio-Rad #500-0205) with BSA as standard. 60µg of total protein was resolved by SDS-PAGE for Western blot analysis using anti-myc monoclonal antibody 9E10 (Santa Cruz Biotechnology). Blots were visualized using Goat anti-mouse immunoglobulin-HRP (GE Healthcare) and the Immunostar detection kit (Bio-Rad). Images were captured using ChemiDoc XRS+ system (Bio-Rad) with Image Lab software.

2.6 Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed essentially as described previously (Rusche et al, 2002) with minor modifications, using either anti-myc antiblody 9E10 (Santa Cruz), or anti-FLAG monoclonal M2 (Sigma F1804). The modifications were that the final immunoprecipitated DNA was collected using the ChIP DNA Clean and Concentrator kit (Zymo Research, D5201) instead of phenol-chloroform extraction/ethanol precipitation, the bead pre-clearing step was increased to 3 hours, and incubation time of antibody with chromatin samples was increased to overnight instead of 1 hour.

2.7 RNA extraction and RT-PCR

Total RNA was extracted as previously described (IYER AND STRUHL 1996) from 35ml cultures at an O.D.600 of approximately 1.0. RNA pellets were redissolved in 300µl DEPC treated water and RNA concentration was measured by UV absorbance at 260nm. Random primed first strand cDNA was synthesized from 500ng total RNA after DNAse treatment (RQ1 DNAse, Promega M6101), using the ProtoScript First Strand cDNA Synthesis Kit (NEB #E6300S). Two sets of primers were designed to detect altered *TFC6* mRNA transcripts in promoter mutants or after Reb1p depletion. Analogous primer sets were used to detect extended *ESC2* transcripts.

2.8 Nucleosome occupancy assay

Changes in relative nucleosome density were determined using a histone H3 ChIP strategy similar to a previously described protocol (SEKINGER *et al.* 2005). Both cell types were processed using the standard ChIP method described above (to yield chromatin with average fragment size ~500 base pairs), then 100ul of each sample was additionally sonicated using a Diagenode Bioruptor sonication system version 1.1 (Cat. No. UCD-600) to yield average fragment size of approximately 200 base pairs (13 cycles, each cycle 30'' on and 30'' off). For each sample (wild-type and *TFC6* Reb1 site promoter mutant), 35ul of this WCE was used for immunoprecipitation with anti-histone H3 antibody (Abcam ab1791). Quantitative PCR (qPCR) was performed as described (Wang et. al 2014) using iQ SYBR Green supermix (Bio-Rad 170-8882). Samples were normalized to the signal generated using primers that amplify a well-positioned nucleosome region within the heterochromatic HMR domain.

Genotypes of all yeast strains used in this study are listed in Table 1. Descriptions of plasmids, and a list of oligonucleotides used are provided in Tables S1 and S2 in the Supporting Information. All yeast strains and plasmids described here will be freely made available upon request.

3. Results

3.1 Ectopic expression of Reb1p increases reporter gene expression driven by the TFC6 promoter.

Previous phylogenic comparison of the *TFC6* promoter region from sequenced *Saccharomyces* species identified a limited number of conserved regions (KLEINSCHMIDT *et al.* 2011). We extended this analysis with sequences from additional available species, and Figure 1A shows the conservation of the *ETC6* site (green shading), plus a sequence just upstream (pink shading) that matches the consensus Reb1p binding site (LIAW AND BRANDL 1994). Analysis of data from multiple high-throughput ChIP-seq studies suggested what appeared to be weak Reb1p binding at this site (RHEE AND PUGH 2011; VENTERS *et al.* 2011). We therefore pursued the hypothesis that Reb1 is the factor that binds to this *TFC6* promoter sequence, and that loss of Reb1 binding is responsible for the phenotypes associated with mutation of the binding site.

To determine if ectopic expression of Reb1p would affect expression from the *TFC6* promoter, we constructed an *ADH1*-promoter driven 3X-FLAG-*REB1* over-expression plasmid (marked with the *HIS3* gene). *S. cerevisiae* encodes another gene encoding a myb-domain called *NSI1,* which has significant homology to *REB1*. Since *NSI1* has been shown to bind to similar DNA sequences and perform some functions

previously attributed to *REB1* (REITER *et al.* 2012), we also constructed an *ADH1*-3X-FLAG-*NSI1* plasmid. We used a previously characterized diploid reporter strain that replaces one copy of the *TFC6* open reading frame (ORF) with the *URA3* ORF (KLEINSCHMIDT *et al.* 2011), so that growth on minimal agar medium lacking uracil could be used as readout of *TFC6* promoter activity. This reporter strain also contains a mutation in the *ETC6* site to relieve TFIIIC mediated negative autoregulation of the promoter driving *URA3* (schematically depicted in Figure 1B). We transformed the reporter strain with either empty vector, or *NSI1* or *REB1* expressing plasmids and selected plasmid containing colonies on media lacking histidine. Individual colonies were next grown in liquid media and plated at approximately 100 cells per plate on media either lacking only histidine, or lacking both histidine and uracil. All transformants grew equivalently on media lacking histidine, showing that maintenance of the expression plasmids themselves did not affect growth (Figure 1B). When plated on media also lacking uracil, slower colony growth was observed when the vector alone or the *NSI1* plasmid was present, due to weak *URA3* expression from the *TFC6* promoter. However, when transformed with the *REB1* expressing plasmid, the average colony size was significantly increased, consistent with the hypothesis that Reb1p binds to and regulates *TFC6* promoter activity.

3.2 Reb1 binds to the TFC6 promoter both in vitro and in vivo.

While these results implicate a role for Reb1 in regulating the *TFC6* promoter, they do not demonstrate a direct interaction. We next cloned the *REB1* DNA binding domain gene sequence into the pET30a(+) over-expression plasmid and purified the

Reb1p DNA binding domain from *E. coli* extracts. We used electrophoretic mobility shift assays (EMSA) to assess the binding of the recombinant protein to different DNA sequences. Figure 2A shows the results of EMSA assays using recombinant Reb1p and three different double stranded oligonucleotide probes. The first panel shows the results using a probe from the ribosomal DNA terminator region, which contains a consensus Reb1p binding site. While the function of this terminator region has recently been shown to be mediated by Nsi1p binding (REITER *et al.* 2012), this sequence had previously been shown to bind Reb1p *in vitro* (MORROW *et al.* 1989; MORROW *et al.* 1990), as was confirmed here. The second panel in Figure 2A shows that the near consensus site in the *TFC6* promoter also binds recombinant Reb1p. The third panel contained oligonucleotides homologous to the coding sequence of *YTA7*, which did not show any detectable Reb1p binding, confirming that our recombinant protein prep does not non-specifically bind DNA.

To confirm *in vivo* binding of Reb1p to the *TFC6* promoter, we constructed yeast strains that contained three copies of the FLAG epitope tag attached to the C-terminus of *REB1*, and performed chromatin immunoprecipitation (ChIP). The recovered DNA was analyzed using primers surrounding the Reb1 consensus site within the *TFC6* promoter, and enrichment of this region compared to no antibody controls was observed for three independently isolated *REB1*-3X-FLAG strains (Figure 2B). Comparable enrichment was not observed at a control locus, a transfer RNA gene on chromosome III.

Given that ectopic overexpression of Tfc6p leads to increased *in vivo* binding of the entire TFIIIC complex to *ETC6* and downregulation of *TFC6* promoter activity (KLEINSCHMIDT *et al.* 2011), and that the Reb1 binding site and *ETC6* sites are adjacent,

we asked whether the binding of either factor affected binding of the other when each was overexpressed ectopically. To address this question, we performed two sets of ChIP experiments. The first used a *TFC1*-3X-FLAG strain to monitor binding of the TFIIIC complex to *ETC6* under conditions of Reb1p overexpression. The second strain contained *REB1*-3X-FLAG, and Tfc6p was overexpressed. The results in Figure 2C, top panel showed no significant difference in TFIIIC binding when Reb1p is overexpressed compared to the same strain transformed with empty vector. Similarly, overexpression of Tfc6p does not alter Reb1p binding as assayed by ChIP. The ChIP samples were also assayed by quantitiative PCR, confirming no significant differences when either protein was overexpressed. These results are consistent with an interpretation that both factors can bind simultaneously, as overexpression of either one does not appear to compete with the other. However, such an interpretation would be tenuous at this point without additional biochemical binding assays.

3.3 Reb1p binding to the TFC6 promoter contributes to proper transcriptional start site selection.

We previously demonstrated that mutation of the Reb1 consensus binding site in the *TFC6* promoter results in reduced Tfc6p expression due to modestly reduced transcript levels (only an approximately 50% reduction), but mainly due to an alteration in the transcriptional start site (TSS) that leads to significantly reduced protein translation (WANG *et al.* 2014). In Reb1 site mutants, transcription from *TFC6* promoter begins farther upstream, resulting in a 5'-extended transcript that contains additional start codons upstream of the canonical *TFC6* start codon, which is likely the cause of the

compromised translation of Tfc6p in these mutants. To further support the interpretation that these effects are mediated by lack of Reb1p binding to this promoter, we created yeast strains with the *GAL1* promoter integrated in place of the *REB1* promoter, and also containing a C-terminal 9X-myc tag on *REB1*. Shifting these strains from galactose to glucose turns off the *GAL1* promoter and metabolically depletes Reb1p. Figure 3A shows a Western blot of the time course of Reb1p-9X-myc depletion in a representative strain. Surprisingly, Reb1p-9X-myc levels persisted even after 8 hours in glucose medium. After overnight incubation, Reb1p was depleted.

We predicted that depletion of Reb1p would phenocopy the promoter site mutation, resulting in the altered TSS (extended at the 5'-end) and reduced Tfc6p translation. Figure 3B shows the results of RT-PCR analysis of *TFC6* transcripts in wild type, promoter mutant, and *GAL1*-promoter-Reb1p depletion strains, grown in either galactose or glucose. Wild type strain DDY3 showed undetectable levels of extended *TFC6* transcripts that begin upstream of the normal TSS, while DDY4300, containing the Reb1 site mutation, contained enriched levels of extended transcripts as we have previously demonstrated (WANG *et al.* 2014). The *GAL1*-promoter-Reb1p depletion strain DDY5248 showed no detectable extended transcript RT-PCR amplification using RNA isolated from cells grown in galactose; however these extended transcripts are enriched after overnight Reb1p depletion in glucose. These results are consistent with Reb1p binding being important in directing proper transcriptional start site selection at this promoter.

We next constructed Reb1p depletion strains that also contained a 3X-FLAG epitope attached to *TFC6* to monitor effects on translation. Figure 3C shows the results of

Western blots of total protein extracts from three independently isolated yeast strains engineered to contain the *GAL1-REB1*-9X-myc depletion allele and a *TFC6*-3X-FLAG allele. Lanes 1-3 contained extracts made from galactose grown cells, and lanes 4-6 are from aliquots of the same cultures after overnight incubation in glucose. Depletion of Reb1p led to reduction of Tfc6p to similar levels as was observed when the Reb1 site is mutated (WANG *et al.* 2014). The results in Figure 3 are consistent with the interpretation that depletion of Reb1p leads to formation of a 5'-extended *TFC6* mRNA transcript, which in turn inhibits proper levels of translation of the *TFC6* coding sequence.

3.4 Mutation of the TFC6 promoter Reb1 site also affects the divergently transcribed ESC2 promoter.

TFC6 and the adjacent *ESC2* genes are divergently transcribed (schematically depicted in Figure 4A). We next asked whether *ESC2* expression was also affected by mutation of the Reb1p binding site or depletion of Reb1p. Analysis of our published transcriptome data comparing wild type and *TFC6* Reb1 site promoter mutants (WANG *et al.* 2014) suggested that *ESC2* mRNAs might also have an altered TSS resulting in a 5'-extension. Figure 4B shows an RT-PCR assay to detect a potential 5'-extension of the *ESC2* mRNA similar to the analysis of *TFC6*. Strain DDY4300 (Reb1p binding site mutant) also showed enrichment of an extended mRNA compared to a wild-type strain (DDY3). Strain DDY5248 also showed enrichment of extended *ESC2* transcripts only after Reb1p depletion by overnight incubation in glucose. These results suggest that Reb1p binding to this divergently transcribed intergenic region is important for proper TSS selection in both directions.

Inspection of the predicted extended *ESC2* mRNA sequence indicated that multiple new AUG start codons would be introduced into the 5'-end *ESC2* transcript, and we hypothesized that this would also lead to inhibition of Esc2p translation as was seen for Tfc6p. We engineered three independent strains that contained the Reb1p depletion allele and an *ESC2*-3X-FLAG allele. Figure 4C shows an anti-FLAG Western blot of the three strains before and after Reb1p depletion, and as predicted, a strong depletion of Esc2p was observed. We were initially surprised at the larger than expected size of the Esc2p-3X-FLAG protein (predicted 56.4 kDa), however, perusal of the literature revealed that several previous studies also reported aberrantly slow SDS-PAGE mobility of epitope tagged Esc2p (OHYA *et al.* 2008; SOLLIER *et al.* 2009; MIMURA *et al.* 2010; YU *et al.* 2010). One possible caveat to these experiments is whether the effects of extended depletion of an essential gene might affect the overall protein loading. However, coomassie staining of the same samples shown in figure 4C show roughly equivalent protein composition before and after Reb1 depletion (Figure S1). Given these results, we conclude that Reb1p binding to the *TFC6* promoter is important for normal TSS selection and proper translation of both *TFC6* and *ESC2*.

3.5 Changes in TSS correlate with altered nucleosome positioning

As an initial test of possible mechanisms of Reb1p function at this intergenic region, we asked whether nucleosome positioning was possibly affected in response to inhibition of Reb1p interaction, by comparing wild type and *TFC6* promoter Reb1 site mutant strains. Numerous studies have mapped nucleosome positions in *S. cerevisiae* (YUAN *et al.* 2005; MAVRICH *et al.* 2008a; KAPLAN *et al.* 2009) showing nucleosome

depleted regions upstream of the +1 nucleosome. A schematic depiction of these mapped nucleosome positions within the *ESC2-TFC6* intergenic region is shown in Figure 5A, using data available through the *Saccharomyces* Genome Database (CHERRY *et al.* 2012). To assay for potential altered nucleosome positioning in wild type versus *TFC6* promoter mutant strains, we used a previously developed nucleosome occupancy assay (SEKINGER *et al.* 2005). We performed ChIP using antibody against histone H3, and analyzed the recovered DNA with primer pairs that amplify the regions designated as ***a***, ***b***, and ***c*** depicted in the figure. The qualitative and quantitative ChIP results shown in Figure 5B demonstrate histone H3 association at the +1 nucleosomes of both *ESC2* and *TFC6* were similar in both strains, however a significant increase in H3 was seen at the annotated nucleosome free region (amplified by primer set ***b***) in the mutant strain. The results are consistent with an alteration of nucleosome positioning and NFR formation at this intergenic region in the absence of Reb1p binding, which we speculate may be involved in the resulting increase of 5'-extended transcripts in both directions.

4. Discussion

DNA binding transcription factors function through various mechanisms, leading to the proper assembly of a preinitiation complex and enhancement of target gene transcription. While a major function of transcription factors involves recruitment of specific proteins to promoters (PTASHNE 2014), studies involving nucleosome positioning also point to a role for DNA-bound transcription factors in the formation and stability of nucleosome depleted regions and statistical positioning of nucleosomes (KORNBERG

1981; MAVRICH *et al.* 2008a). The yeast general regulatory factor Reb1 has been implicated not only as a mediator of nucleosome positioning (HARTLEY AND MADHANI 2009; YEN *et al.* 2012), but also has been shown to block the progression of RNA Polymerase II (COLIN *et al.* 2014). Our results described here suggest that Reb1 may utilize both of these activities in regulating the proper transcriptional start sites at the divergently transcribed *ESC2-TFC6* locus.

Our previous work demonstrated that mutation of the now confirmed Reb1p binding site (Figures 1 and 2) within the *TFC6* promoter results in only a modest (approximately 50%) reduction of *TFC6* mRNA levels(KLEINSCHMIDT *et al.* 2011; WANG *et al.* 2014), but more significantly results in an increase in the level of extended upstream transcription initiation. This 5'-extended transcript is poorly translated, as Tfc6 protein levels are reduced approximately 15-fold in the mutants (WANG *et al.* 2014), most likely due to the inclusion of multiple additional upstream AUG codons in the extended mRNA molecules. Recent studies have shown significant natural heterogeneity in yeast TSSs leading to mRNAs with differing translatability (ROJAS-DURAN AND GILBERT 2012; MALABAT *et al.* 2015). Transcription factors such as Reb1p may be required to prevent or minimize the frequency of aberrant 5'-extended transcripts from being produced. Regulation of TSS selection might be especially important at bidirectional promoters, as we demonstrate here that compromising Reb1p binding to the *TFC6-ESC2* intergenic region (by either mutation of the binding site or Reb1p depletion) has similar effects on both genes (Figures 3 and 4), that being an increase in the level of 5'-extended mRNAs and subsequent severe effect on translation of both gene products.

It is well documented that upstream open reading frames (uORFs) can have regulatory effects on mRNA translation (SONENBERG AND HINNEBUSCH 2009; MCGEACHY AND INGOLIA 2016). In order to assess the possibility that such effects could be occurring at this locus, we performed an extended Clustal analysis to see if uORFs 5'-to both *TFC6* and *ESC2* were conserved among the budding yeast (Figure S2). Aside from one location in the upstream region of *ESC2*, there is no conservation of the location of additional start codons introduced in the 5'-extended transcripts for either gene. Potential peptides encoded by such uORFs are also not conserved, again suggesting no real regulatory role for extended transcripts. We therefore conclude that the role of Reb1p is to maintain the proper transcriptional start site to prevent random inclusion of aberrant start codons into the 5'-untranslated region of both mRNAs.

Previous studies have shown that Reb1p has a significant role in establishing the positioning of the NDRs at promoters (HARTLEY AND MADHANI 2009; YEN *et al.* 2012), and appears to specifically set the border of the -1 nucleosome, preferentially at divergently transcribed promoters (KOERBER *et al.* 2009). Consistent with these past results, we find that the normal NDR upstream of *TFC6* has an increased histone H3 ChIP signal in the Reb1p binding site mutant (Figure 5), which correlates with the observed aberrant transcription starts for both *TFC6* and *ESC2*. Interestingly, a genome-scale mapping of TSSs in yeast shows unexpected heterogeneity in transcript 5'-ends even in wild type cells, with many genes showing transcripts containing extended 5'-UTRs that might not be translated properly due to the presence of upstream short open reading frames (MALABAT *et al.* 2015). We suggest that the altered nucleosome occupancy observed when Reb1p binding is inhibited at the *TFC6* promoter increases the

frequency of heterogeneous TSSs at this locus, leading to the drastic decrease in both Tfc6 and Esc2 protein levels.

Another layer of transcript quality control that might be at play in this region is the noted ability of DNA-bound Reb1p to act as a roadblock to Pol II transcription (COLIN *et al.* 2014). Analysis of the data from the TSS study cited above (MALABAT *et al.* 2015) reveals that even in wild type cells, a fraction of *TFC6* and *ESC2* transcripts are initiated upstream of the normal TSS; such mRNAs are thought to be degraded by the nonsense mediated decay pathway. We speculate that transcripts initiating on the "wrong side" of the Reb1p binding site may be additionally kept in check by upstream initiating Pol II encountering the Reb1p roadblock.

Regulation of gene expression in eukaryotic cells is a varied and complex process with many layers. While many studies use transcript levels as a sole readout of gene expression (often measured by RT-PCR of a small internal mRNA sequence), it has been noted that translational regulation by TSS selection may be a considerably underappreciated mechanism of protein level control (ROJAS-DURAN AND GILBERT 2012). Our data presented here demonstrate that disruption of a single DNA-protein interaction at a divergently transcribed genomic region can have significant effects on transcription start site usage, and lead to consequences in protein-level expression that are far greater in magnitude than the modest effects on mRNA levels.

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We thank Raphyel Rosby for providing *GAL*-promoter depletion templates, primer sequences, and advice on their use. We also thank Wes Frey for construction and verification of the *REB1* and *NSI1* ectopic expression plasmids during his lab rotation. Finally, we thank Richard Kleinschmidt and Kimberly LeBlanc for construction of previously unpublished strain DDY4441 used in this study. This work was funded by grant MCB-1329446 from the National Science Foundation.

Table 1 – *S. cerevisiae* strains used and generated in this study.

Name	Genotype	Source
DDY3	*MAT**a** his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1*	J. Rine
DDY3630	*MAT**a** his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 ETC6 wild type*	Donze Lab
DDY4107	*MAT**a** his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 TFC6:3XFLAG:KanMX*	Donze Lab
DDY4300	*MAT**a** his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 TFC6 Reb1 site promoter mutant*	Donze Lab
DDY4441	*MAT**a** his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 REB1-3XFLAG-KanMX*	Donze Lab
DDY4521	*MATα/MAT**a** ade2/ADE2 his3/his3 leu2/leu2 LYS2/lys2Δ trp1/trp1 ura3/ura3 ETC6-TFC6/etc6 (C>G)-tfc6[ORF]Δ::URA3[ORF]*	Donze Lab
DDY5032	*MATα his3-11 leu2-3,112 lys2Δ _trp1-1 ura3-1 REB1-9Xmyc-TRP1*	This Study
DDY5061	*MAT**a** ade2-1 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 etc6Δ::URA3 REB1-9XMyc-TRP1*	This Study
DDY5238	*MAT**a** his3-11 leu2-3,112 trp1-1 ura3-1 REB1-3XFLAG-KanMX TFC6 promoter wild-type*	This Study
DDY5239	*MATα his3-11 leu2-3,112 trp1-1 ura3-1 REB1-3XFLAG-KanMX TFC6 promoter wild-type*	This Study
DDY5240	*MATα/MAT**a** ade2/ADE2 his3-11/his3-11 leu2-3,112/leu2-3,112 lys2Δ/lys2Δ trp1-1/trp1-1 ura3-1/ura3-1 REB1-9XMYC-TRP1/REB1-9XMYC-TRP1 etc6Δ::URA3*	This Study
DDY5242	*MATα/MAT**a** ade2-1/ADE2 his3-11/his3-11 leu2-3,112/leu2-3,112 lys2Δ/lys2Δ _trp1-1/trp1-1 ura3-1/ura3-1 KanMX-pGAL1-REB1-9XMYC-TRP1/REB1-9XMYC-TRP1 etc6Δ::URA3*	This Study
DDY5248	*MATα his3-11 leu2-3,112 lys2Δ trp-11 ura3-1 KanMX-pGAL1-REB1-9XMYC-TRP1*	This Study
DDY5290	*MATα his3-11 leu2-3,112 lys2Δ trp-11 ura3-1 NatMX6-pGAL1-REB1-9XMYC-TRP1*	This Study
DDY5293-5295	*MATα his3-11 leu2-3,112 lys2Δ trp-11 ura3-1 NatMX6-pGAL1-REB1-9XMYC-TRP1 ESC2-3X-FLAG-kanMX*	This Study
DDY5302, 5304	*MAT**a** his3-11 leu2-3,112 lys2Δ trp-11 ura3-1 NatMX6-pGAL1-REB1-9XMYC-TRP1 TFC6-3X-FLAG-kanMX*	This Study
DDY5303	*MATα his3-11 leu2-3,112 lys2Δ trp-11 ura3-1 NatMX6-pGAL1-REB1-9XMYC-TRP1 TFC6-3X-FLAG-KanMX*	This Study

All strains are isogenic to *S. cerevisiae* W303-1A

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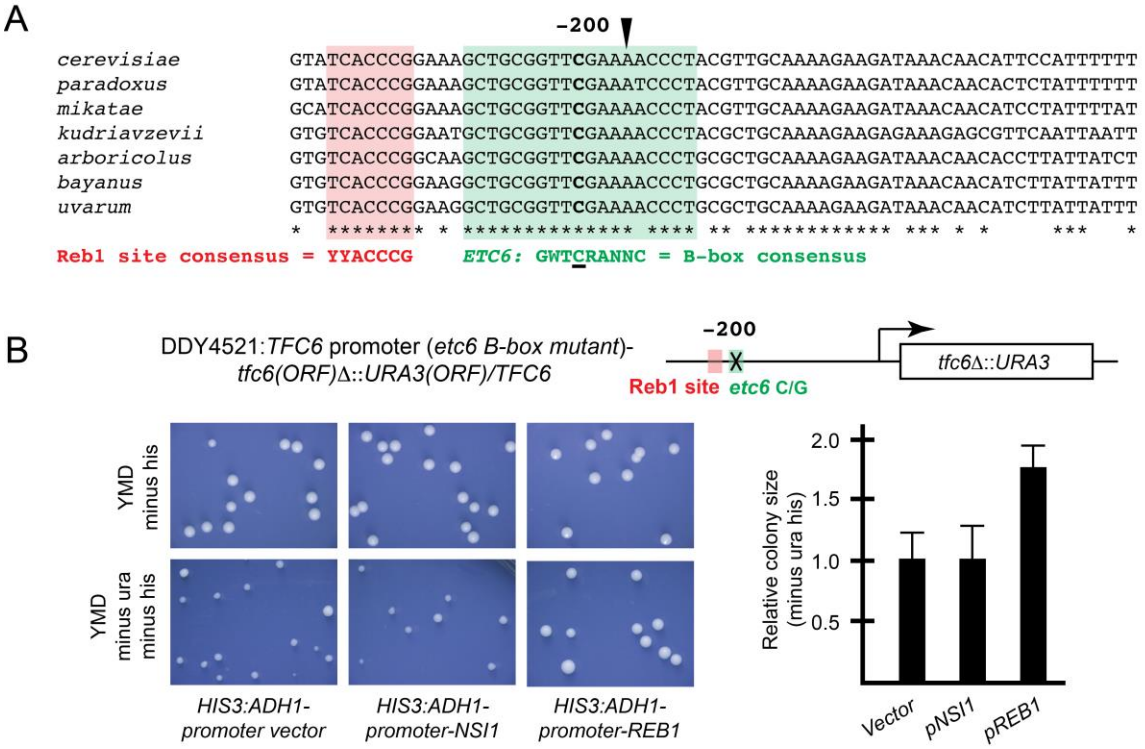

Figure 1 Over-expression of Reb1p increases expression from the *TFC6* promoter. (A) Sequence alignment of the *TFC6* promoter region from seven different budding yeast species. The pink shaded block shows conservation of the consensus Reb1 binding site and the green shaded block shows the conserved *ETC6* site containing the TFIIIC binding B-box consensus. The black arrowhead indicates the -200 site (relative to the start codon) of the *TFC6* promoter. (B) Overexpression of Reb1p enhances *URA3* expression driven by the *TFC6* promoter. DDY4521 is a diploid strain with the *URA3* coding sequence replacing one copy of the *TFC6* coding sequence. The *ETC6* site has been mutated to inhibit TFIIIC binding (green) and the Reb1 site is indicated by the pink shading. This strain was transformed with empty vector (pDD1242), or the same vector overexpressing *NSI1* (pDD1250) or *REB1* (pDD1251). Overexpression of Reb1p but not Nsi1p resulted in faster colony growth on medium lacking uracil and histidine, but not on plates lacking

only histidine. Error bars represent the standard deviation of colony diameters based on the measurement of at least 32 colonies for each plasmid transformant.

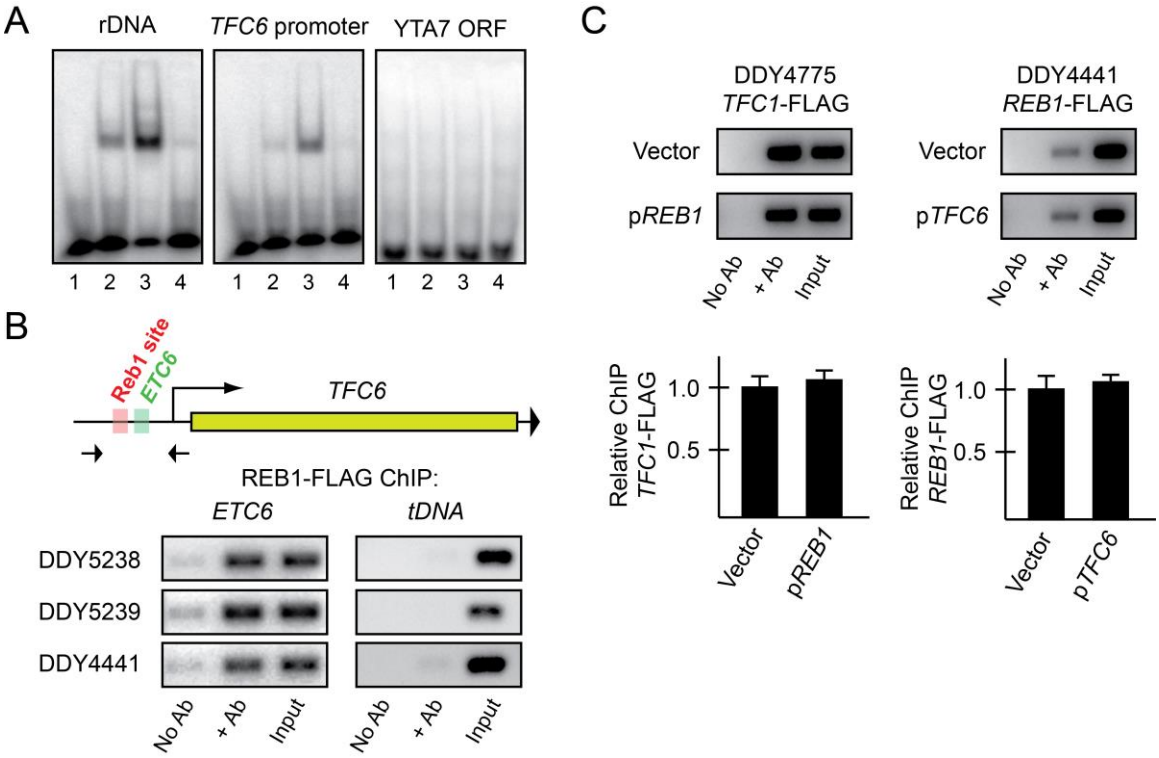

Figure 2 Reb1p binds to the *TFC6* promoter region both *in vitro* and *in vivo*. (A) EMSA using purified recombinant Reb1p incubated with radiolabeled probes corresponding to the yeast rDNA terminator site (positive control), the *TFC6* promoter site, and the *YTA7* ORF (negative control). Lanes 1-3 in each panel contained increasing amounts of Reb1p (0ng, 200ng, 500ng) and lane 4 was incubated with 500ng Reb1p plus 100-fold excess unlabeled probe. EMSA results showed that purified recombinant Reb1p DNA binding domain specifically binds to the *TFC6* promoter sequence upstream of *ETC6* and to the rDNA enhancer site, but not to the *YTA7* ORF. (B) Chromatin immunoprecipitation of Reb1. Yeast strains DDY5238, DDY5239, and DDY4441 contain a 3X-FLAG epitope tag at the C-terminus of the *REB1* coding sequence. Anti-FLAG antibody was used to immunoprecipitate crosslinked target DNA. Samples were amplified with oligonucleotides DDO-705 and -706 surrounding the Reb1p binding site at *ETC6*. The

Reb1 signal was enriched for all three yeast strains at *TFC6* promoter, verifying that Reb1p is bound to the *TFC6* promoter *in vivo*. As a negative control, the ChIP samples were amplified with Oligonucleotides DDO-59 and -60, which amplify a transfer RNA gene on chromosome III. (C) Overexpression of either Reb1p or Tfc6p does not influence binding of each other. DDY4775 contains the FLAG epitope tagged *TFC1*, and DDY4441 contains FLAG tagged *REB1*. Both strains were transformed with an empty vector or a vector overexpressing either Reb1p or Tfc6p. Plasmid transformed isolates were processed for ChIP, and the recovered DNA was quantitated by real-time PCR. The percentage of input control of each transformant is shown on the bar graph. Overexpression of either protein (Reb1p or Tfc6p) did not significantly affect the binding of the other (Tfc6p as part of the entire TFIIIC complex) at the *TFC6* promoter.

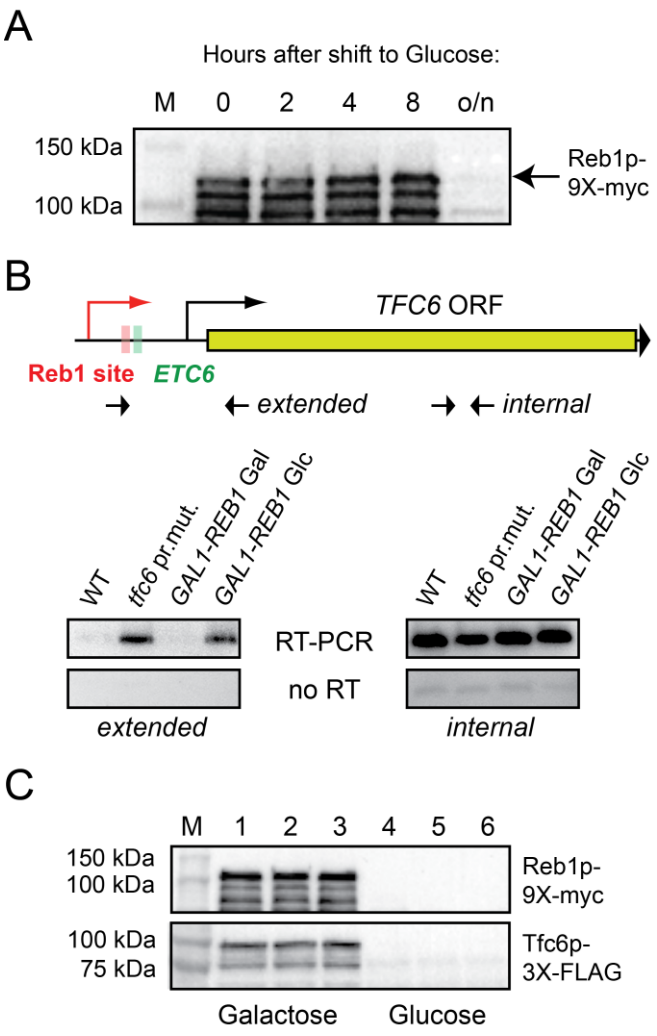

Figure 3 Depletion of Reb1p phenocopies *TFC6* promoter mutation. (A). Western blot of Reb1p depletion time course post glucose shift. The *GAL1* promoter-*REB1*-9X-myc yeast strain DDY5248 was shifted to YPD medium and incubated for 2 hrs., 4 hrs., 8 hrs., or overnight to deplete Reb1p. (B). RT-PCR of *TFC6* to detect extended mRNAs. Black bent arrow shows normal transcriptional start site and red arrow shows the approximate start region of extended transcripts. Small black arrows show the relative position of two sets of PCR primers, with the upstream set specific for extended transcripts. Compromised Reb1p binding by either promoter mutation or Reb1p depletion leads to increased production of extended *TFC6* transcripts. (C) Western blots detecting Reb1p-

9X-myc and Tfc6p-3X-FLAG for three independently isolated yeast strains (lanes 1-3 are DDY5302, DDY5303, and DDY5304) grown in YPGal media. Lanes 4-6 are the same strains shifted to YPD media overnight. M=molecular weight markers.

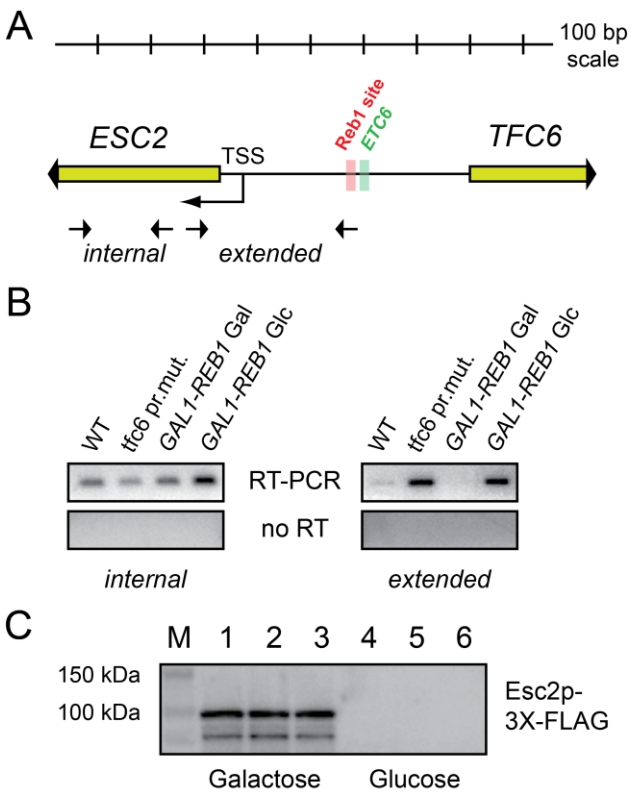

Figure 4 Compromised Reb1 binding also affects *ESC2* at both transcriptional and translational level. (A) Schematic diagram depicting the relative genomic location of *ESC2* and *TFC6*. The black arrow (at TSS) shows the normal transcription start site of *ESC2* and small black arrows underneath show the position of two sets of primers used for RT-PCR. (B). RT-PCR of *ESC2* transcripts. The extended transcript is significantly enriched when Reb1p binding to the *TFC6* promoter is compromised by either binding site mutation or Reb1p depletion. (C). Western blot of Esc2p-3X-FLAG after Reb1p depletion. Lanes 1-3 contain protein extracted from three independently isolated yeast strains grown in YPGal, and lane 4-6 are the same three yeast strains grown overnight in YPD to deplete Reb1p. Strains used were DDY5293, DDY5294 and DDY5295.

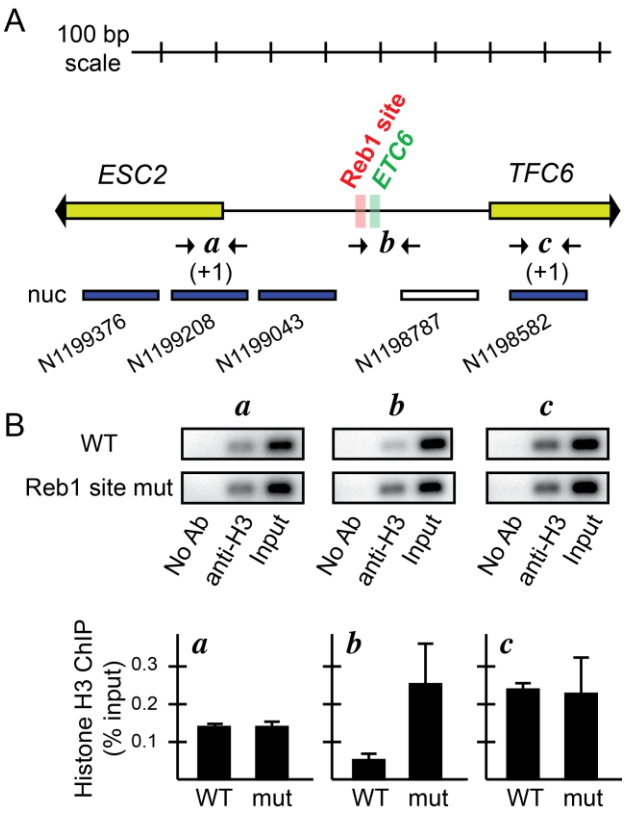

Figure 5 Alteration of nucleosome positioning at the *ESC2-TFC6* intergenic region after Reb1p depletion. (A). Schematic depiction of mapped nucleosome positions at the intergenic region *ESC2-TFC6.* Nucleosomes are annotated (the letter N followed by the *S. cerevisiae* chromosome IV position) according to Mavrich et al., 2008, from data accessible from the *Saccharomyces* Genome Database. The nucleosome just upstream of the *TFC6* ORF (N1198787) is un-shaded to indicate a much weaker signal in the cited reference (Kaplan et al., 2009), and is possibly part of the NDR. Relative locations of primer sets ***a***, ***b***, and ***c*** used to assay histone H3 enrichment are designated by the black arrows. (B). Histone H3 enrichment along the intergenic region in wild type versus Reb1p binding site mutant analyzed by chromatin immunoprecipitation. (C) Relative quantification of immunoprecipitated DNA by real-time PCR. Error bars represent the standard deviation of triplicate samples assayed.